

REMARKS

Following the Examiner's request, Applicants have withdrawn claims 3 and 9-26 from further consideration. However, Applicants respectfully request that the Examiner consider these claims after the claims under examination are found allowable. See the discussion below. Claims 28-32 were previously cancelled.

Upon entry of the above amendment, claims 1, 2, 4-8, and 27 will be pending and under examination. Applicants respectfully request that the Examiner reconsider this application in view of the following remarks.

Rejection under 35 U.S.C. § 103

Claims 1, 2, 4-8, and 27 are rejected for obviousness on two grounds, each of which is traversed below.

I

The Examiner rejects claims 1, 2, and 5-8 for obviousness relying on Hwang et al., US Patent 5,905,089 (Hwang) and Baba et al., US Patent 6,123,943 (Baba). Independent claims 1 and 5 will be discussed first.

Claim 1 covers treating hepatitis C virus (HCV) infection with a sesquiterpene lactone compound. Claim 5 covers treating HCV infection with a sesquiterpene lacton compound featuring γ -lactone fused with a 10-membered ring.

Hwang describes a group of sesquiterpene lactone compounds that inhibit NF- κ B activity. Nowhere is it taught or even suggested in this reference treating HCV infection with these compounds.

Baba describes that 1,2,3,4-tetrahydroisoquinoline compounds can be used to treat a large number of diseases, including viral hepatitis, via inhibiting NF-kB activity. It does not teach or suggest any sesquiterpene lactone compound, let alone using it to treat any viral hepatitis, not to mention HCV infection.

Yet, the Examiner takes the position that one skilled in the art would have been motivated to use the NF-kB inhibitors described in Hwang, i.e., sesquiterpene lacton

As shown in the passage quoted above, the size of the genus of diseases listed in Baba is very large. In this connection, Applicants would like to bring to the Examiner's attention that the law is well established that rejection of a claimed species in light of a prior art genus is not appropriate where the prior art does not disclose a small recognizable class of species. See *In re Ruschig*, 343 F.2d 965, 974, 145 USPQ 274, 282 (CCPA 1965). Like the prior art at issue in *Ruschig*, Baba describes a very large genus of diseases, not a small recognizable class of diseases. Pursuant to the *Ruschig* holding, the Examiner's rejection of claims 1 and 5, covering treatment of a specific disease (i.e., HCV infection) is not appropriate.

In addition, Applicants would like to point out that Baba merely describes a group of 1,2,3,4-tetrahydroisoquinoline compounds that inhibit NF- κ B activity. It does not show whether these NF- κ B inhibitors are effective in treating any of the listed diseases, let alone HCV infection. Given the high unpredictability in the medicine field, it is uncertain as to whether or not HCV infection can be effectively treated by inhibiting NF- κ B activity. To Applicants' best knowledge, no effective anti-HCV infection drugs have yet been developed via discovering their inhibitory effects against NF- κ B activity. In other words, one skilled in the art, in view of Baba, would not have predicted that 1,2,3,4-tetrahydroisoquinoline compounds can be effectively use to treat HCV infection.

Even if 1,2,3,4-tetrahydroisoquinoline compounds could be effectively use to treat HCV infection, one still would not have predicted success of using sesquiterpene lactone compounds described in Hwang in place of 1,2,3,4-tetrahydroisoquinoline compounds to treat HCV infection. In this regard, Applicants have submitted herewith as "Exhibit A" a copy of a scientific publication, i.e., Aubin et al., J. Neurochem., 1998, 71:1635-1642 ("Aubin"), which discloses that different NF- κ B inhibitors may have totally different effects in treating the same disease. More specifically, Aubin describes that aspirin and salicylate protect against MPTP-induced dopamine depletion in mice and that dexamethasone, a much more potent NF κ B inhibitor, is totally ineffective against MPTP toxicity in this dopamine depletion mouse model. See Abstract and page 1641, left

compounds, to treat viral hepatitis via inhibiting NF-kB activity as taught in Baba. See the Office Action, page 3, lines 10-13.

Applicants do not agree. Baba describes a very large number of diseases ranging from inflammatory diseases to autoimmune diseases to viral diseases. See column 3, lines 1-3. It states, in relevant part, that:

That is, the drug of the present invention is effective for the treatment and prevention of diseases such as rheumatoid arthritis, systemic lupus erythematosus, systemic scleroderma, Behcet disease, periarteritis, ulcerative colitis, Crohn disease, active chronic hepatitis, glomerular nephritis and the like various autoimmune diseases; and osteoarthritis, gout, atherosclerosis, psoriasis, atopic dermatitis, pulmonary diseases with granuloma, various intractable diseases in which inflammatory symptoms such as of various types of encephalitis are the basis of the morbid state, endotoxin shock, sepsis, inflammatory colitis, diabetes, acute myelocytic leukemia, pneumonia, heart transplantation, encephalomyelitis, anorexia, acute hepatitis, chronic hepatitis, drug induced hepatic injury, alcoholic hepatitis, viral hepatitis, jaundice, hepatic cirrhosis, hepatic insufficiency, atrial myxoma, Castleman syndrome, multiple myeloma, Rennert T lymphomatosis, mesangial nephritis, renal cell carcinoma, cytomegaloviral hepatitis, cytomegaloviral retinopathy, adenoviral cold syndrome, adenoviral pharyngoconjunctival fever, adenoviral ophthalmia, AIDS and the like. Column 8, lines 26-46.

This reference does not specifically disclose HCV infection. To come up with treating HCV infection, one would have to first select viral hepatitis from the list and then select HCV infection from viral hepatitis. In other words, it appears to be the Examiner's position that one would have been motivated to select a species, i.e., HCV infection, from the genus of diseases listed in Baba to arrive at the claimed invention. Applicants would like to point out that, to determine whether there is motivation to select a species from a prior art genus, the Examiner must consider factors including the size of the genus and the predictability of the technology. See MPEP 2144.08.II.4.¹

¹ It states that "[t]o address this key issue [i.e., whether it would have been obvious to select the claimed species from the disclosed prior art genus], Office personnel should consider all relevant prior art teachings, focusing on the following, where present ... (a) Consider the Size of the Genus ... (e) Consider the Predictability of the Technology ..."

column, first paragraph. Clearly, whether NF- κ B is effective in treating a certain disease is highly unpredictable.

In short, using NF- κ B to treat HCV infection is highly unpredictable. To this end, one skilled in the art would have had no motivation to select a specific species (i.e., HCV infection) from the large genus of diseases listed in Baba, let alone replacing the NF- κ B inhibitors described in Baba with those described in Hwang.

Given the large size of the genus of diseases listed in Baba and the high unpredictability of the utilities relating to NF- κ B, Applicants submit that one skilled in the art, in view of Hwang and Baba, would not have been motivated to treat HCV infection with sesquiterpene lactone compounds to arrive at the invention covered by claims 1 and 5. In other words, claims 1 and 5 are not rendered obvious by these two references.

For the same reasons set forth above, claim 2, dependent from claim 1, and claims 6-8 and 27, dependent from claim 5, are also not rendered obvious by these two references.

II

The Examiner rejects claims 4 and 27 for obviousness relying on Hwang, Baba, and Tan et al., Nature Review, 2002, 1: 867-881 (Tan).

Claims 4 and 27 depend from claims 1 and 5, respectively. Their patentability resides at least in part in treating HCV infection with a sesquiterpene lactone compound. As discussed above, Hwang and Baba, taken alone or in combination, do not teach or suggest such treatment. Tan also fails to do so. It merely discloses treating HCV infection using intron A, which is a protein, not a sesquiterpene lactone compound.

As none of Hwang, Baba, and Tan teaches or suggests treating HCV infection with a sesquiterpene lactone, any combination of these three references also fails to do so. As such, claims 4 and 27 are not rendered obvious by these three references.

Elected Claims 3 and 9-26

In response to the restriction requirements dated January 2, 2008 and March 18, 2008, Applicants elected claims 1-27 for prosecution and also elected as a species a

sesquiterpene lacton compound featuring γ -lactone fused with a 10-membered ring.

Applicants further pointed out that claims 1, 2, 4-8, and 27 read on this species.

In the Office Action, the Examiner has only considered claims 1, 2, 4-8, and 27 and rejected these claims for obviousness relying on Hwang, Baba, and Tan. On the other hand, he has withdrawn from further consideration claims 3 and 9-26, which cover non-elected species.

The law is clear that "should the examiner determine that the elected species is allowable, the examination of the Markush-type claim will be extended [to non-elected species]." See MPEP 803.02. As discussed above, the Examiner errs in relying on Hwang, Baba, and Tan to reject claims 1, 2, 4-8, and 27, covering the elected species, and the elected species is actually allowable. Pursuant to the above-quoted MPEP guidance, Applicants respectfully request that the Examiner proceed to extend the examination to non-elected species, which are covered by claims 3 and 9-26.

CONCLUSION

It is believed that all of the pending claims have been addressed. However, the absence of a reply to a specific rejection, issue or comment does not signify agreement with or concession of that rejection, issue or comment. In addition, because the arguments made above may not be exhaustive, there may be reasons for patentability of any or all pending claims (or other claims) that have not been expressed. Finally, nothing in this paper should be construed as an intent to concede any issue with regard to any claim, except as specifically stated in this paper, and the amendment of any claim does not necessarily signify concession of unpatentability of the claim prior to its amendment.

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Respectfully submitted,

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EXHIBIT A

Aspirin and Salicylate Protect Against MPTP-Induced Dopamine Depletion in Mice

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Abstract: The neurotoxic effects of the dopamine-selective neurotoxin MPTP (15 mg/kg, s.c.), in mice, were totally prevented by systemic administration of salicylate (ED_{50} = 40 mg/kg, i.p.), aspirin (ED_{50} = 60 mg/kg, i.p.), or the soluble lysine salt of aspirin, Aspegic (ED_{50} = 80 mg/kg, i.p.). The protective effects of aspirin are unlikely to be related to cyclooxygenase inhibition as paracetamol (100 mg/kg, i.p.), diclofenac (100 mg/kg, i.p.), ibuprofen (20 mg/kg, i.p.) and indomethacin (100 mg/kg, i.p.) were ineffective. Dexamethasone (3–30 mg/kg, i.p.), which, like aspirin and salicylate, has been reported to inhibit the transcription factor NF- κ B, was also ineffective. Aspirin or salicylate (100 μ M) had no effect on dopamine uptake into striatal synaptosomes or on monoamine oxidase B activity. The neuroprotective effects of salicylate derivatives could perhaps be related to hydroxyl radical scavenging. This was suggested by the fact that hydroxylated metabolites of salicylate (2,3- and 2,5-dihydrobenzoic acid) were recovered in brain tissue following the combined administration of MPTP and aspirin to a greater extent than following aspirin alone. The surprising neuroprotective effects of aspirin in an animal model of Parkinson's disease warrant further clinical investigation. **Key Words:** Aspirin—Salicylic acid—1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine—Mouse—Neurotoxicity—Parkinson's disease model.

J. Neurochem. **71**, 1635–1642 (1998).

Since the chance discovery of the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in humans (Langston and Ballard, 1983), it has been thought that a similar type of environmental toxin might be responsible for idiopathic Parkinson's disease and reasoned that an understanding of the neurotoxic effects of MPTP might lead to prevention of this debilitating disorder. MPTP is metabolized to the 1-methyl-4-phenylpyridinium ion (MPP⁺) by monoamine oxidase (MAO) B (Langston et al., 1984), and this highly toxic metabolite is selectively taken up into dopaminergic neurons via the dopamine (DA) transporter (Snyder and D'Amato, 1986). MPP⁺ is a mitochondrial toxin that selectively inhibits complex 1 of the respiratory

chain (Cleeter et al., 1992), leading evidently to energy compromise and to the production of potentially cytotoxic free radicals (Jenner, 1991; Adams et al., 1993; Bowling and Beal, 1995). Accumulating evidence suggests that oxidative stress is also a feature of Parkinson's disease neuropathology (Dexter et al., 1986; Jenner, 1991; Fahn and Cohen, 1992; Schapira, 1994; Bowling and Beal, 1995), and mitochondrial complex 1 deficiency is also a feature of this disease (Mizuno et al., 1989; Schapira et al., 1990). Numerous studies in rodents or primates (including, of course, the original observation in humans) have shown that MPTP produces a selective lesion of the nigrostriatal DA system that closely mimics the neuropathological and symptomatic sequelae of Parkinson's disease. Blockade either of MAO-B (Rose et al., 1989) or of DA uptake (Bradbury et al., 1985) is known to protect against the neurotoxic effects of MPTP in laboratory animals, although recent clinical trials with the selective MAO-B inhibitor deprenyl have met with mixed success (Olanow et al., 1995; Parkinson Study Group, 1996). This type of clinical approach does not attack the neurotoxic process itself [although antiapoptotic and free radical scavenging effects have recently been attributed to deprenyl (Tatton et al., 1994; Gerlach et al., 1996)] but aims rather to prevent the metabolism of a putative (and unknown) MPTP-like molecule to a toxic metabolite.

Several arguments suggest that the neurotoxic effects of MPTP (or MPP⁺) might be mediated via free radical production. This may result from disturbances

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Abbreviations used: DA, dopamine; DHBA, dihydroxybenzoic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine (serotonin); HVA, homovanillic acid; MAO, monoamine oxidase; MPP⁺, 1-methyl-4-phenylpyridinium ion; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 3-MT, 3-methoxytyramine; PEA, phenylethylamine.

in oxygen metabolism produced by complex 1 inhibition or from diverse effects of MPTP and MPP⁺ on free radical-generating processes (for reviews, see Gerlach et al., 1991; Tipton and Singer, 1993; Zang and Misra, 1993). MPTP decreases glutathione levels and increases the levels of reactive oxygen species and the degree of lipid peroxidation in mouse brain slices *in vitro* and increases the levels of reactive oxygen species in mouse brain *in vivo*. MPTP neurotoxicity *in vitro* is reduced by glutathione (Sriram et al., 1997). *In vitro* studies have shown that MPP⁺ neurotoxicity can be reduced by vitamin E, vitamin C, coenzyme Q, and mannitol (but not by superoxide dismutase, catalase, allopurinol, or dimethyl sulfoxide) (Akaneya et al., 1995). β -Carotene, vitamin C, and *N*-acetylcysteine partially protect against the neurotoxic effects of MPTP in mice (Perry et al., 1985), as do nicotinamide, coenzyme Q, and the free radical spin trap *N*-tert-butyl- α -(sulphophenyl)nitron (Schulz et al., 1995a).

Chuih and others have demonstrated that MPTP results in the formation of the highly toxic hydroxyl radical *in vivo*. Hydroxyl radical formation was monitored in dialysis studies by the salicylate detection method (Chuih et al., 1992, 1993; Obata and Chuih, 1992). Hydroxyl radicals react with salicylate to form 2,3- and 2,5-dihydroxybenzoic acid (DHBA). MPTP increases the formation of both 2,3- and 2,5-DHBA *in vivo*. If hydroxyl radicals are interacting with salicylate, then of course salicylate is also mopping up hydroxyl radicals. If indeed these are the agents through which MPTP exerts its neurotoxicity, we reasoned that salicylate should provide some degree of neuroprotection in the MPTP model. We found that salicylate totally protects against the neurotoxic effects of MPTP and that this property is shared by acetylsalicylate (aspirin) and a lysine salt formulation of aspirin, Aspegic, but not by other cyclooxygenase inhibitors.

MATERIALS AND METHODS

Animals and tissues

The MPTP studies were conducted on male C57Bl/6 mice (weighing 20–25 g; Iffa Credo, France). *In vitro* studies on DA uptake or MAO-B activity were conducted using brain tissue from male Sprague–Dawley rats (weighing 180–250 g; Iffa Credo, France). Animals were housed in a controlled environment (light/dark cycles of 12 h with lights on from 7 a.m. to 7 p.m., temperature of $21 \pm 2^\circ\text{C}$) with food and water *ad libitum*.

Assay of striatal DA, serotonin [5-hydroxytryptamine (5-HT)], and their related metabolites

Mice were killed by decapitation. Striata were dissected out, frozen, weighed, and stored at -80°C until analysis. 5-HT, DA, homovanillic acid (HVA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxytyramine (3-MT), and 5-hydroxyindoleacetic acid (5-HIAA) were quantified by HPLC with electrochemical detection. Frozen tissues were sonicated in 800 μl of 0.05 *M* HClO₄ containing 0.5 *mM* EDTA, 2 *mM* sodium metabisulfite, and 3,4-DHBA (final

concentration, 1 ng/50 μl) as the internal standard. After centrifugation, 50 μl of the supernatant was injected onto the liquid chromatographic column using a refrigerated (4°C) autoinjector (Wisp 712; Waters, Milford, MA, U.S.A.). Separation was achieved at room temperature. The HPLC system consisted of a pump and a stainless steel separation column (0.46×7 cm) packed with Ultrasphere XL ODS C18 (particle size, 3 μm ; Beckman, Fullerton, CA, U.S.A.). The mobile phase contained 0.1 *M* NaH₂PO₄, 1 *mM* EDTA, 2.5 *mM* octanesulfonic acid, and 7% CH₃CN, pH 3.4. The flow rate was 0.9 ml/min. Electrochemical detection was carried out by means of an amperometric detector (model 460; Waters) with a glassy carbon working electrode and an Ag/AgCl reference electrode. The detector potential was set at 0.8 V versus the reference electrode. Concentrations of each compound were calculated using a computing integrator (Maxima; Waters) with reference calibration curves obtained after injection of standards.

Assay of striatal salicylate, 2,3-DHBA, and 2,5-DHBA

Salicylate and its hydroxylated metabolites were quantified using the methods described by Giovanni et al. (1995). Mice were killed by decapitation. Striata were dissected out, frozen, weighed, and stored at -80°C until analysis. Frozen tissues were sonicated in 150 μl of 0.05 *M* HClO₄ containing 0.5 *mM* EDTA and 2 *mM* sodium metabisulfite. After centrifugation, 50 μl of the supernatant was injected onto the liquid chromatographic column using a refrigerated (4°C) autoinjector (Wisp 712; Waters). Separation was achieved at room temperature using a stainless steel separation column (0.46×7 cm) packed with Ultrasphere XL ODS C18 (particle size, 3 μm ; Beckman). The mobile phase contained 0.1 *M* NaH₂PO₄, 1 *mM* EDTA, 2.25 *mM* octanesulfonic acid, and 5% CH₃CN, pH 3.2. The flow rate was 0.9 ml/min. For 2,3- and 2,5-DHBA the detection was carried out by means of an amperometric detector (model 460; Waters). The detector potential was set at 0.8 V versus the reference electrode. Salicylate was detected with an ultraviolet detector set at 300 nm. Concentrations of each compound were calculated using a computing integrator (Maxima; Waters) with reference calibration curves obtained after injection of standards.

Determination of MAO-A and MAO-B activities

Rat brains were homogenized in 20 volumes of 0.1 *M* sodium phosphate buffer (pH 7.4) at 4°C . MAO-A and MAO-B activities were assayed as previously described (Curet et al., 1996) using [¹⁴C]5-HT (final concentration, 125 μM) as the specific MAO-A substrate or phenyl[¹⁴C]ethylamine ([¹⁴C]PEA; final concentration, 8 μM) as the specific MAO-B substrate.

In vitro measurement of [³H]DA uptake in rat brain synaptosomes

Rat striatal synaptosomes were prepared at 4°C by homogenization in 40 volumes of 0.32 *M* sucrose with 10 strokes of a Teflon/glass homogenizer. After centrifugation at 2,000 *g* for 10 min, the supernatant was centrifuged at 10,000 *g* for 20 min, and the pellet was resuspended in 0.32 *M* sucrose (2 ml) and recentrifuged with 0.8 *M* sucrose (8 ml). The resulting pellet was resuspended in 6 ml of buffer (10 *mM* HEPES, 147 *mM* NaCl, 5 *mM* KCl, 2 *mM* MgCl₂, 2 *mM* CaCl₂, and 10 *mM* glucose, adjusted to pH 7.4). All subsequent operations were at 25°C . Fifty-microliter aliquots

(~50 μ g of protein per well) were distributed into 96-well glass fiber filter plates (MAFCNOB; Millipore SA), and drugs were added in a further 50 μ l of the above HEPES buffer following a 10-min incubation. [3 H]DA (final concentration, 400 nM) was added 10 min before filtration on a Multiscreen system, and synaptosomes were washed twice with 200 μ l of HEPES buffer. After direct application of a solid scintillator (MeltiLex; Wallac) to the 96-well glass fiber filter plates, radioactivity was counted in a microplate scintillation counter (MicroBeta 1450 Trilux; Wallac).

Body temperature measurement

Mice core temperature was measured at different times after MPTP or salicylate administration using a small rectal probe and Thermistor thermometer with minimal immobilization stress.

Statistics

The statistical significance of the data was evaluated by ANOVA followed by Dunnett's (homogeneous variances) or Kruskal-Wallis (heterogeneous variances) test.

Chemicals and drugs

[14 C]5-HT creatinine sulfate (1.8–2.2 GBq/mmol) was supplied by Amersham (Buckinghamshire, U.K.). [14 C]PEA hydrochloride (1.8–2.2 GBq/mmol), [3 H]DA (1,365 GBq/mmol), and Biofluor were purchased from New England Nuclear (Boston, MA, U.S.A.). Salicylic acid, aspirin, diclofenac, dexamethasone, ibuprofen, 2,3-DHBA, 2,5-DHBA, 3,4-DHBA, DA, noradrenaline, DOPAC, 5-HIAA, 3-MT, 5-HT creatinine sulfate, PEA hydrochloride, and 2,5-diphenyloxazole were supplied by Sigma (St. Louis, MO, U.S.A.). Toluene, EDTA, and ethyl acetate were purchased from Labosi (Paris, France). MPTP was obtained from Research Biochemicals International (Natick, MA, U.S.A.). The analytical grade buffers $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, and perchloric acid were purchased from Merck (Darmstadt, Germany). Aspegic, paracetamol, and indomethacin were synthesized in the Chemistry Department at Synthelabo.

Drug treatments

Aspirin, dexamethasone, paracetamol, diclofenac, and indomethacin were administered intraperitoneally as a suspension in 0.5% Methocel gel plus 0.5% Tween 80 (wt/wt) in a volume of 10 ml/kg. Salicylic acid, Aspegic, ibuprofen, MPTP, and 2,3- and 2,5-DHBA were administered in saline in a volume of 10 ml/kg. Doses always refer to the free base and are expressed in mg/kg of body weight.

RESULTS

Effects of MPTP on DA, 5-HT, and their metabolites in striatum

Mice received graded doses of MPTP (10, 20, 30, 40, and 50 mg/kg, s.c.) and were killed 2 days later. As illustrated in Fig. 1A, MPTP decreased the striatal levels of DA, DOPAC, HVA, and 3-MT in a dose-dependent manner without affecting the levels of 5-HT and 5-HIAA. The maximal decrease of DA levels was obtained with a dose of 50 mg/kg, s.c. Higher doses of MPTP induced mortality. A dose of 15 mg/kg MPTP was chosen for further studies. The time course of the effects of MPTP (15 mg/kg, s.c.) on striatal DA, DOPAC, and 3-MT levels is shown in Fig.

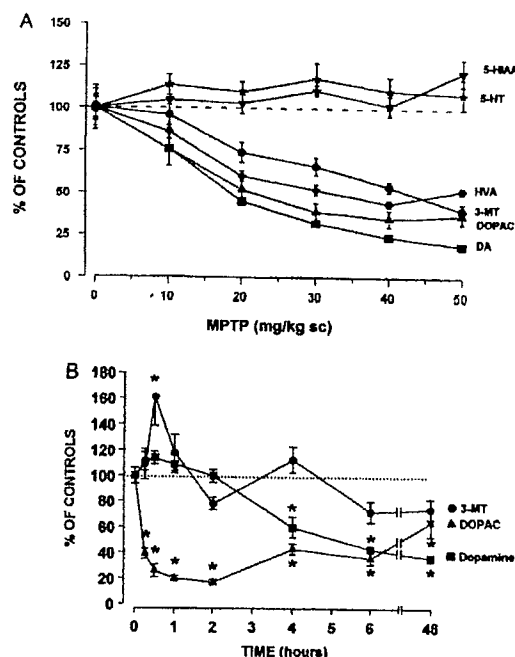


FIG. 1. A: Effects of administration of graded doses of MPTP on striatal levels of DA, DOPAC, HVA, 3-MT, 5-HT, and 5-HIAA in mouse brain. The animals were killed 2 days after administration of MPTP (10–50 mg/kg, s.c.). Data are mean \pm SEM (bars) values ($n = 8-9$), as a percentage of variations versus controls. Control levels (in pg/mg of tissue) were as follows: DA, $13,440 \pm 1,147$; DOPAC, $1,377 \pm 505$; 3-MT, 585 ± 109 ; 5-HT, 442 ± 105 ; and 5-HIAA, 328 ± 97 . **B:** Time course of the effects of MPTP (15 mg/kg, s.c.) on striatal levels of DA, DOPAC, and 3-MT. Animals were killed at defined times following MPTP administration.

1B. As described by others (Pileblad et al., 1985), MPTP produced a rapid and sustained decrease in striatal DOPAC levels that was maximal 2 h after MPTP injection. DA levels more gradually declined over the 48-h period. DA depletion was sustained for at least 2 weeks (see, e.g., Fig. 3). MPTP also produced a transient increase in striatal 3-MT levels within the first hour following injection. No mortality was observed with this dose of MPTP for up to 2 weeks after MPTP injection.

Protective effect of aspirin, Aspegic, and salicylate against MPTP-induced DA depletion

In a first experiment, mice were pretreated with different doses of aspirin, Aspegic, and salicylate 1 h before administration of MPTP (15 mg/kg, s.c.). Striatal DA levels were measured 2 days later. As illustrated in Fig. 2, aspirin, Aspegic, and salicylate each prevented MPTP-induced DA depletion in a dose-related manner with ED_{50} values of 60, 80, and 40 mg/kg, i.p., respectively. This protection was total at the highest dose of each drug. The total protective effect of the highest dose of salicylate (100 mg/kg, i.p.) was

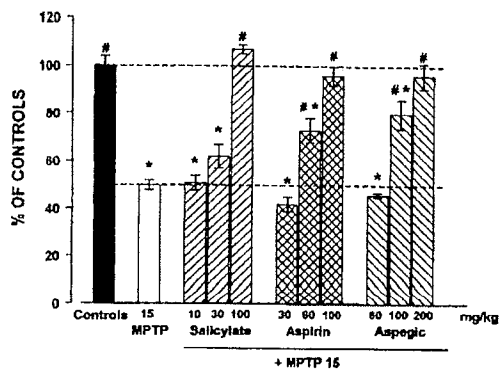


FIG. 2. Effects of systemic intraperitoneal administration of graded doses of salicylate, aspirin, and Aspegic on depletion of DA in striatum of mice induced by MPTP. The animals received a single administration of salicylate (10, 30, or 100 mg/kg, i.p.), aspirin (30, 60, or 100 mg/kg, i.p.), or Aspegic (60, 100, or 200 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 2 days later. Data are mean \pm SEM (bars) values ($n = 6$). Control DA levels were $14,310 \pm 719$ pg/mg of tissue. * $p < 0.01$ compared with control; * $p < 0.01$ compared with MPTP group.

maintained in mice killed 2 weeks after administration of MPTP (15 mg/kg, s.c.; Fig. 3). Whereas salicylate (100 mg/kg, i.p.) totally blocked the effects of MPTP at 15 mg/kg, s.c., it only partially protected (50%) against the effects of a higher dose (40 mg/kg, s.c.) of the toxin (Fig. 4). To study the time course of the effect of aspirin (60 mg/kg, i.p.), the drug was administered at different times before and after a single dose of MPTP (15 mg/kg, s.c.). As illustrated in Fig. 5, significant protection was observed when aspirin was administered from 2 h before MPTP and for up to 2 h following MPTP administration. The maximal

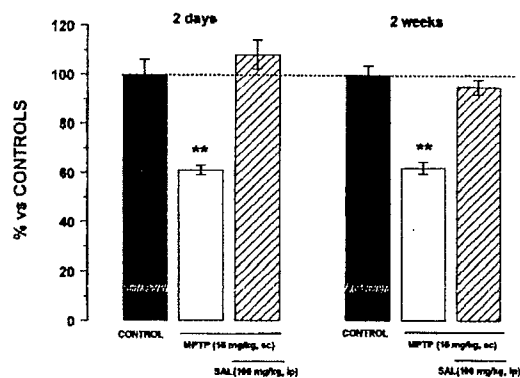


FIG. 3. Protective effects of salicylate (SAL) on depletion of DA in striatum of mice induced by MPTP 2 days or 2 weeks after treatments. The animals received a single administration of SAL (100 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 2 days or 2 weeks later. Data are mean \pm SEM (bars) values ($n = 6$). Control DA levels were $11,799 \pm 704$ and $15,452 \pm 544$ pg/mg of tissue at 2 days and 2 weeks, respectively. ** $p < 0.01$ compared with the respective control.

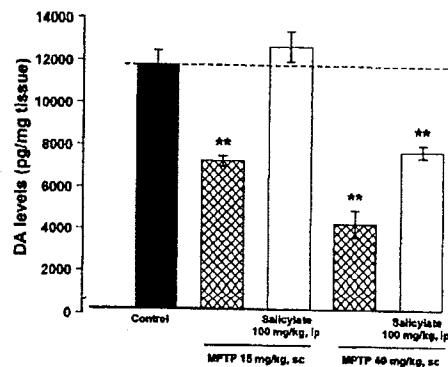


FIG. 4. Effects of salicylate on depletion of DA in striatum of mice induced by different doses of MPTP. The animals received a single administration of salicylate (100 mg/kg, i.p.) 1 h before MPTP (15 or 40 mg/kg, s.c.) and were killed 2 days later. Data are mean \pm SEM (bars) values ($n = 6$). Control DA levels were $11,799 \pm 704$ pg/mg of tissue. ** $p < 0.01$ compared with control.

protection was observed when aspirin was given concomitantly or 1 h after MPTP. No protection was seen when aspirin was delayed for >4 h after MPTP administration.

Aspirin (100 mg/kg, i.p.), salicylate (100 mg/kg, i.p.), or Aspegic (200 mg/kg, i.p.) per se had no effect on striatal levels of DA 2 days after dosing (data not shown).

Acute effects of aspirin and MPTP on DA and its metabolites in rat striatum

The neurotoxic effects of MPTP as revealed by chronic DA depletion are not observed until several hours after MPTP administration. However, more immediately after MPTP injection, effects on striatal DA turnover can be observed that reflect the toxin's more acute effects on DA uptake and metabolism, e.g., Fig.

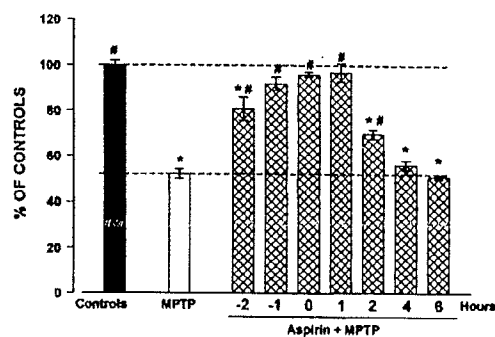


FIG. 5. Time course of protective effects of aspirin on MPTP-induced depletion in DA level in mouse striatum. The animals received a single administration of aspirin (100 mg/kg, i.p.) at different times before or after MPTP (15 mg/kg, s.c.) and were killed 2 days later. Data are mean \pm SEM (bars) values ($n = 6$). Control DA levels were $14,450 \pm 879$ pg/mg of tissue. * $p < 0.01$ compared with control; * $p < 0.01$ compared with MPTP group.

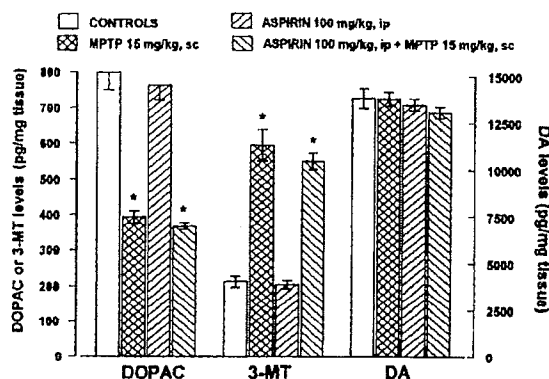


FIG. 6. Acute effects of MPTP (15 mg/kg, s.c.) and aspirin (100 mg/kg, i.p.) alone or combined on striatal DA metabolism. Vehicle or aspirin was injected 1 h before MPTP injection. The effects of MPTP on striatal levels of DA, DOPAC, and 3-MT were measured 30 min after injection of MPTP or 1 h 30 min after injection of aspirin. Data are mean \pm SEM (bars) values ($n = 8$). * $p < 0.05$, ** $p < 0.01$ compared with control.

1B. MPTP (15 mg/kg, s.c.) decreased striatal DOPAC and increased striatal 3-MT levels 30 min after injection, without at this early stage reducing striatal DA levels (Fig. 6).

Aspirin per se (100 mg/kg, i.p.) had no effect on DA, 3-MT, or DOPAC levels (Fig. 6) 1 h 30 min after injection, and when administered 1 h before MPTP did not modify the MPTP-induced acute increase in 3-MT or decrease in DOPAC levels.

Effect of MPTP on salicylate and DHBA striatal levels after administration of aspirin

It has already been shown that MPTP increases the cerebral hydroxylation of systemically administered salicylate (Chiueh et al., 1992, 1993). When aspirin (100 mg/kg, i.p.) was administered to mice, high levels of salicylate and its hydroxylated metabolites, 2,3- and 2,5-DHBA, were detected in striatal samples 1 h 30 min later (Table 1). When MPTP (15 mg/kg, s.c.) was given 1 h following aspirin administration, 30 min before the animals were killed, a marked increase in the levels of the hydroxylated metabolites, 2,3-DHBA ($p < 0.01$) and 2,5-DHBA ($p < 0.05$), was observed (Table 1). The ratios of 2,3-DHBA/salicylate and 2,5-

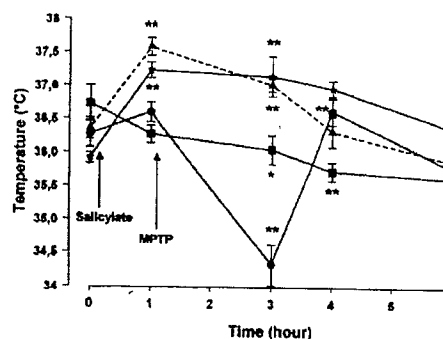


FIG. 7. Time course of effects of MPTP (15 mg/kg, s.c.) and salicylate (100 mg/kg, i.p.) alone or in combination on the core temperature of mice. Salicylate (100 mg/kg, i.p.) or vehicle was administered 1 h before MPTP (15 mg/kg, s.c.): controls (■), MPTP (●), salicylate (▲), and salicylate plus MPTP (★). Data are mean \pm SEM (bars) values ($n = 6$). * $p < 0.05$, ** $p < 0.01$ versus respective control (core temperature before salicylate or vehicle administration).

DHBA/salicylate were increased by 158 and 66%, respectively, by MPTP administration.

Systemic administration of the hydroxylated metabolites of salicylate (2,3- and 2,5-DHBA (100 mg/kg, i.p.) was without effect on the DA depletion induced by MPTP (data not shown).

Effects of MPTP, salicylate, aspirin, and Aspegic on body temperature of mice

As illustrated in Fig. 7, MPTP (15 mg/kg, s.c.) induced a transient decrease in core temperature with a maximal effect of -2°C , 2 h after administration. This decrease was blunted by salicylate administered 1 h before MPTP. Salicylate alone or in combination with MPTP produced a small but significant increase in core temperature from 1 h up to 6 h after administration.

Lack of effect of salicylate, aspirin, and Aspegic on synaptosomal DA uptake and on MAO-A and MAO-B activities

Salicylate or aspirin (100 μM) had no significant effect on striatal [^3H]DA uptake in rat striatal synaptosomes or on MAO-A or MAO-B activity in rat brain homogenates. DA uptake values were 64.0 ± 5 , 57.6

TABLE 1. Effect of MPTP on hydroxyl radical formation as assessed by breakdown of salicylate to 2,3- and 2,5-DHBA in mouse striatum after administration of aspirin

Treatment	Salicylate (nmol/g)	2,3-DHBA (pmol/g)	2,5-DHBA (pmol/g)	2,3-DHBA/salicylate	2,5-DHBA/salicylate
Aspirin	43 ± 8	12.6 ± 5.8	148 ± 32	0.24 ± 0.07	3.56 ± 0.42
Aspirin + MPTP	57 ± 10^a	40.1 ± 11^b	355 ± 79^c	0.62 ± 1.1^b	5.91 ± 0.46^c

The animals received a single administration of aspirin (100 mg/kg, i.p.) 1 h before MPTP (15 mg/kg, s.c.) and were killed 30 min later. Data are mean \pm SEM values ($n = 8$).

^a Not significant, ^b $p < 0.05$, ^c $p < 0.01$ compared with aspirin group.

TABLE 2. Effects of diclofenac, indomethacin, ibuprofen, paracetamol, and dexamethasone on reduction in striatal DA levels in mice produced by MPTP

	Diclofenac (100 mg/kg, i.p.)	Indomethacin (100 mg/kg, i.p.)	Ibuprofen (20 mg/kg, i.p.)	Paracetamol (100 mg/kg, i.p.)	Dexamethasone		
					3 mg/kg, s.c.	10 mg/kg, s.c.	30 mg/kg, s.c.
Control	13,763 \pm 832	13,763 \pm 832	11,799 \pm 704	13,805 \pm 219	14,480 \pm 359	14,480 \pm 359	14,480 \pm 359
MPTP (15 mg/kg, s.c.)	5,640 \pm 305 ^a	5,640 \pm 305 ^a	7,257 \pm 249 ^a	7,545 \pm 137 ^a	7,570 \pm 310 ^a	7,570 \pm 310 ^a	7,570 \pm 310 ^a
MPTP + drug	4,957 \pm 907 ^a	7,522 \pm 268 ^a	6,954 \pm 113 ^a	8,047 \pm 152 ^a	8,600 \pm 395 ^a	8,188 \pm 352 ^a	8,657 \pm 238 ^a

Drugs were administered 1 h before MPTP, and the mice were killed 2 days later. DA levels are mean \pm SEM values ($n = 8$ striata), in pmol/mg of tissue.

^a $p < 0.001$ versus control.

± 6 , and 62.7 ± 4 pmol/min/mg of protein in the control, aspirin (100 μ M), and salicylate (100 μ M) groups, respectively. MAO-A activity values were 0.130 ± 0.06 , 0.123 ± 0.05 , and 0.126 ± 0.05 nmol/min/mg of tissue in control, aspirin (100 μ M), and salicylate (100 μ M) groups, respectively. The corresponding values for MAO-B activity were 0.108 ± 0.06 , 0.108 ± 0.05 , and 0.105 ± 0.06 nmol/min/mg of tissue.

Effect of cyclooxygenase inhibitors and dexamethasone on MPTP-induced DA depletion

Paracetamol (100 mg/kg, i.p.), ibuprofen (20 mg/kg, i.p.), indomethacin (100 mg/kg, i.p.), diclofenac (100 mg/kg, i.p.), or dexamethasone (3, 10, and 30 mg/kg, s.c.) was administered 1 h before MPTP (15 mg/kg, s.c.). None of these drugs decreased the neurotoxic effect of MPTP (Table 2).

DISCUSSION

Salicylate, aspirin, and its soluble lysine salt, Aspegic, are able to protect totally against the neurotoxic effects of MPTP in mice. Protection by salicylate was maintained for at least 2 weeks after MPTP administration and is not a transient effect. The neuroprotective effects of aspirin were fully maintained when aspirin was administered 1 h after MPTP treatment, and significant (but reduced) protection was still observed with an administration delay of 2 but not 4 h. The process with which aspirin interacts is thus manifest within the first 4 h following MPTP administration. During this time hydroxyl radical production is evident (Chiueh et al., 1992, 1994; Obata and Chiueh, 1992). The maximal decrease in mitochondrial complex I activity is observed ~ 1 h following MPTP administration in mice (Sriram et al., 1997). None of these drugs affected DA uptake or MAO-B activity, and their neuroprotective effects are therefore related to interference with a neurotoxic process and not to blockade of MPTP metabolism or MPP⁺ uptake into dopaminergic terminals. Within the first hours of administration, MPTP results in an acute increase in striatal 3-MT levels coupled with a reduction in striatal DOPAC levels, likely

related to its immediate effects on DA uptake and metabolism, as shown by Pileblad et al. (1985). These acute effects of MPTP were totally unaffected by salicylate, suggesting that salicylate does not interfere with the cerebral entry or metabolism of MPTP itself. Salicylate was less protective against a higher dose of MPTP, suggesting some type of competitive effect between salicylate and the neurotoxic effector. The effects of salicylate cannot be explained in terms of hypothermia, and indeed salicylate appeared to produce slight hyperthermic effects per se. The hypothermic effects of MPTP are likely related to its acute neurotoxic effects, and their antagonism by salicylate is a symptomatic manifestation of its neuroprotective action. It seems unlikely that cyclooxygenase inhibition explains the neuroprotective effects of aspirin. Salicylate, which was also effective in this model, is only a weak inhibitor of cyclooxygenase (Mitchell et al., 1994), and other reference cyclooxygenase inhibitors [acetaminophen (paracetamol), diclofenac, ibuprofen, and indomethacin (Mitchell et al., 1994)] were ineffective against the neurotoxic effects of MPTP.

Aspirin and salicylate (at millimolar concentrations) have both been reported to inhibit the activation of the transcription factor NF- κ B in various in vitro models (Kopp and Ghosh, 1994; Grilli et al., 1996). Diverse noxious cellular stimuli free NF- κ B from an endogenous inhibitor, allowing translocation of free NF- κ B from the cytoplasm to the nucleus. NF- κ B then binds to DNA and activates several genes involved in inflammatory and immune responses. Some of these gene products, for example, tumor necrosis factor- α , may exert cytotoxic effects by switching on apoptotic self-destruct programs (Wright et al., 1992; Vaux and Strasser, 1996). Apoptosis has been reported to be a feature of MPP⁺-related cytotoxicity (Mochizuki et al., 1994). The effects of MPTP or MPP⁺ on NF- κ B activation do not appear to have been studied, although increased translocation of NF- κ B has been observed in Parkinson's disease brain (Hunot et al., 1997). Both aspirin and salicylate block the glutamate-induced activation of NF- κ B in rat cerebellar granule cells and furthermore block the neurotoxicity of glutamate on

cerebellar granule cells and in hippocampal slices (Grilli et al., 1996). As indomethacin does not block NF- κ B activation or glutamate toxicity in vitro (Grilli et al., 1996) and did not protect against the neurotoxic effects of MPTP, it is possible that NF- κ B inhibition is somehow involved in the MPTP neurotoxic cascade and in the protective effects of aspirin. However, dexamethasone, which is a much more potent (nanomolar) inhibitor of NF- κ B activation in similar in vitro models (Auphan et al., 1995), was totally ineffective against MPTP toxicity.

Nitric oxide synthase inhibitors have been reported to block the neurotoxic effects of MPTP (Schulz et al., 1995b; Przedborski et al., 1996). Aspirin does inhibit nitric oxide synthase at high concentrations (IC₅₀ of ~1 mM), but salicylate is without effect (Amin et al., 1995), and it seems unlikely that nitric oxide synthase inhibition explains these neuroprotective effects. It has also been suggested that the protective effects of certain nitric oxide synthase inhibitors may in fact be related to their additional ability to inhibit MAO-B (Di Monte et al., 1997).

Salicylate is an effective hydroxyl radical trapping agent, and indeed an increase in levels of the hydroxylated metabolites of salicylate following MPTP coadministration was initially used to show that MPTP generates hydroxyl radicals in vivo (Chiueh et al., 1992, 1993; Obata and Chiueh, 1992). Aspirin also effectively traps hydroxyl radicals (Halliwell et al., 1987) and is rapidly metabolized to salicylate following systemic administration (Gaspari et al., 1989). Following the administration of aspirin, our results show that salicylate and its hydroxylated metabolites can be found in the mouse brain. The production of these hydroxylated metabolites is increased by MPTP, confirming that MPTP generates hydroxyl radicals that react with salicylate derived from aspirin. Hydroxyl radical scavenging activity is thus one possible explanation for the neuroprotective effects of aspirin and salicylate. Other free radical scavengers have been reported to be effective in the MPTP model, although it has to be said that their neuroprotective effects have not been as impressive (Perry et al., 1985; Akaneya et al., 1995; Schulz et al., 1995a). It should also be mentioned that a very potent hydroxyl radical scavenger, dimethyl sulfoxide (Gaspari et al., 1989), does not protect against the neurotoxic effects of MPP⁺ in vitro (Akaneya et al., 1995).

At this stage, and without further experiments designed specifically to address the question, it is probably wise to reserve judgment on the mechanism of neuroprotective action of aspirin and salicylate, although one can at least rule out cyclooxygenase inhibition. It is nevertheless surprising and exciting that these two drugs are able to prevent completely neurotoxic effects in this animal model of Parkinson's disease—an observation that merits close clinical attention.

Aspirin is known to produce toxic effects at high doses in humans (for example, acidosis and gastroin-

testinal bleeding). In these animal experiments, the results suggest some form of competition between aspirin and the toxic effector produced by MPTP, i.e., the higher the dose of MPTP, the more aspirin is needed to combat its effects. In the animal studies, dopaminergic toxicity is produced by a bolus injection of a single high dose of MPTP, and very high doses of aspirin or salicylate are necessary to combat its effects. In the clinical situation one would hope that the long time course of the degenerative process in Parkinson's disease reflects chronic toxicity of lower levels of any putative MPTP-like neurotoxin. If this were the case, it is possible that relatively low repeated doses of aspirin or salicylate might be able to slow disease progression.

In clinical trials, aspirin has been shown to be of clear benefit in the prevention of Stroke (Antiplatelet Trialists' Collaboration, 1994), a factor generally attributed to its beneficial effects on platelet aggregation. The accumulating reported neuroprotective effects of aspirin perhaps hint at a more direct effect on neuronal resistance. It has also been noted that there is an inverse correlation between antiinflammatory treatment (including the use of aspirin) and Alzheimer's disease (Breitner et al., 1994). Both aspirin and salicylate block glutamate-induced neurotoxicity in vitro (Grilli et al., 1996) and MPTP-induced neurotoxicity in vivo, via mechanisms that are clearly unrelated to the classical primary effect of aspirin, cyclooxygenase inhibition. There appears to be a hidden aspect of aspirin and salicylate pharmacology whose further characterization may lead to the clearer definition of neurotoxic processes and to the development of novel treatments in various central degenerative diseases.

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